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Preliminary Report on the Mutagenicity of Carbaryl

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16. ABSTRACT

Carbaryl has been reported to cause point mutations in bacteria, Drosophila, and mammalian cells in vitro; unscheduled DNA snythesis in human cells in culture; and chromosome effects (including spindle effects) in plants, mammalian cells in vitro, and animals. Although there are inadequacies in these studies, the results when considered together are strongly suggestive that carbaryl may have the intrinsic ability to act as a mutagen. It should be noted that because carbaryl appears to act as a weak mutagen in the experimental test systems, it is likely to act as a weak mutagen in humans as well. To cause heritable effects in humans, however, carbaryl and/or an active metabolite(s) must reach the germinal tissue. Evidence that carbaryl reaches the mammalian gonad is considered suggestive. Adverse gonadal effects, e.g., abnormal sperm morphology (Degraeve et al. 1976), reduction in the number of spermatogonia and spermatozoa in the seminiferous tubules, (Kitagawa et al. 1977) reduced sperm motility (Shtenberg and Rybakova 1968), and decreased fertilization index (Collins et al. 1971) have been reported in rodents exposed to carbaryl. In addition, abnormal sperm head morphology has been reported in workers with known exposure to carbaryl (Wyrobek et al. 1980). Therefore, carbaryl may have the potential to act as a germ-cell mutagen.

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THE REPRODUCTIVE EFFECTS ASSESSMENT GROUP'S PRELIMINARY REPORT ON THE MUTAGENICITY OF CARBARYL

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PURPOSE

The Reproductive Effects Assessment Group (REAG) conducted an extensive review of published and unpublished data regarding the mutagenicity of carbaryl (Sevin) at the request of the Special Pesticide Review Division of the Environmental Protection Agency's Office of Pesticide Programs as part of their Rebuttable Presumption Against Registration (RPAR). The acceptability of the test results was determined and the mutagenic potential of carbaryl was assessed.

OVERVIEW

Mutagenic chemicals are recognized as posing a potential risk to human health because of their ability to cause heritable changes in genes and chromosomes. Such germline changes, for example, can lead to spontaneous abortions, birth defects, or the accumulation of deleterious mutations in the human gene pool. In addition, somatic mutations may be involved in the etiology of cancer.

The primary objective of a mutagenicity risk assessment is to determine the potential of a chemical to cause heritable germline effects in man (Environmental Protection Agency's "Proposed Guidelines for Mutagenicity Risk Assessment," Federal Register 45:74984, November 1980). Positive responses have been reported in three gene mutation test systems (bacteria, Drosophila, and mammalian cells in culture) using carbaryl (McCann, 1980 personal communication, Cook et al. 1977, Rashid 1978, Egert and Greim 1976, Brzheskii 1972, Ahmed et al. 1977a). In addition, the results of cytogenetic tests suggest that carbaryl may induce chromosomal aberrations in mammalian cells in culture (Ishidate and Odashima 1977, Kazarnovskaya and Vasilos 1977); carbaryl

has been shown to cause spindle effects in plants (Wuu and Grant 1966, Amer 1965, Amer et al. 1971, Amer and Farah 1968, Brankovan 1972) and may cause spindle effects in rodents (Vasilos et al. 1975a, b) and in human cells in vitro (Vasilos et al. 1972, Shpirt 1975); and carbaryl has been shown to cause unscheduled DNA synthesis, which is indicative of primary DNA damage, in cultured human cells (Ahmed et al. 1977b). Although there are inadequacies in the available studies, the results, when considered together, are strongly suggestive that carbaryl may act as a mutagen.

To cause heritable effects in humans, however, a chemical with intrinsic mutagenicity must reach the germinal tissue. Evidence that carbaryl and/or its active metabolites reaches mammalian gonads is suggestive. Adverse gonadal effects, e.g., abnormal sperm morphology (Degraeve et al. 1976), reduction in the number of spermatogonia and spermatozoa in the seminiferous tubules, (Kitagawa et al. 1977) reduced sperm motility (Shtenberg and Rybakova 1968), and decreased fertilization index (Collins et al. 1971) have been reported in rodents exposed to carbaryl. In addition, abnormal sperm head morphology has been reported in workers with known exposure to carbaryl (Wyrobek et al. 1980). Therefore, carbaryl may have the potential to act as a germ cell mutagen.

Further experiments are needed, however, to better characterize the mutagenic risk of carbaryl (e.g., gene mutation tests in Drosophila or mammalian cells in vitro describing a dose-response, and data demonstrating alkylation or radiolabeling in germ cell DNA of intact animals). It should be noted that carbaryl is not a potent mutagen in the reported studies, and probably acts as a weak mutagen only.

It is well-established that nitrosocarbaryl, a nitroso derivative of carbaryl, is a potent mutagen in bacteria. A dose-related increase in mutants

above the spontaneous mutation frequency has been observed (Blevins et al. 1977, Elespuru et al. 1974, Marshall et al. 1976, Seiler 1977, Greim et al. 1977, and Uchiyama et al. 1975). Also, some investigators have shown that nitrosocarbaryl can cause primary DNA damage as manifested by the ability to strongly induce mitotic gene conversion in yeast (Siebert and Eisenbrand 1974) and by the detection of DNA strand breaks and unscheduled DNA synthesis in cultured human cells (Regan et al. 1976). Although, there is no available evidence regarding the ability of nitrosocarbaryl to reach or affect the mammalian gonads, nitrosocarbaryl is intrinsically mutagenic and thus likely to cause somatic mutations in humans which may be involved in the etiology of cancer. Some studies indicate that nitrosocarbaryl given orally to rats results in the development of carcinomas in the forestomach (Eisenbrand et al. 1976, Lijinsky and Taylor 1976). Because the in vivo formation of this compound in the human stomach from nitrites and carbaryl in the diet is conceivable (Rickard 1979), nitrosocarbaryl should be included as a potential parameter in the toxicological consideration of carbaryl.

REVIEW OF EXPERIMENTAL EVIDENCE ON THE MUTAGENICITY OF CARBARYL

EVIDENCE CONCERNING GENE MUTATIONS
(Studies summarized in Tables 1 and 2)

Gene mutations are defined as point mutations and interstitial deletions. Point mutations are limited to intralocus changes that affect one or a few base-pairs out of about 10^3 to 10^5 base-pairs which constitute a gene (e.g., base-pair substitutions, base-pair additions or deletions). An interstitial deletion is a minor deletion which may involve several gene loci, a single gene locus, or a part of a gene locus (Flamm 1977).

Before discussing the results of the mutagenicity tests, it should be

emphasized that a negative result ir a mutation test does not preclude the mutagenic activity of a compound. The specificity of a test system for the detection of a particular type of mutagen and the level of sensitivity should be considered. Furthermore, the original form of a chemical may not be mutagenic but rather one of its metabolites may be an active mutagen. Thus, a compound inactive in the absence of metabolic activation in an *in vitro* test should also be assessed in the presence of mammalian microsomal enzymes (e.g., S9 fraction). More importantly, the experimental assay may have been conducted improperly. For example, concurrent negative and positive controls must be included. In addition, before investigating the mutagenic effect of any agent, it is essential to determine the quantitative dependence of cell killing of the chemical dose to be tested. Not only should an appropriate concentration range be examined but an adequate number of tests should be performed to confirm a negative or positive result.

Bacteria

Bacterial tests in which carbaryl has been reported as negative include back mutations in *Bacillus subtilis*, forward mutations in *Haemophilus influenzae*, and back and forward mutations in *Escherichia coli* (Table 1). Several of these reports (DeGiovanni-Donnelly et al. 1968, Egert and Greim 1976, Fahrig 1974, Fiscor and Lo Piccolo 1972, Shirasu et al. 1976, Uchiyama et al. 1975) contained no or only a brief description of the protocol. Most of these negative reports do not describe the toxicity of the test material (see Table 1). The importance of toxicity measurements is that excessive cell killing by the chemical could result in the elimination of revertants or mutants (i.e., false negative result) or that an observation of a negative result and no toxicity may be due to the fact that the test material did not enter the cell (i.e., a no-test classification). In addition, because it has

TABLE 1. POINT MUTATION TESTS USING BACTERIA

Reference	Test System	Strain	Activation System	Concentration	Result	Comment
Ashwood-Smith et al. 1972	Reversion to tryptophan prototrophy in *E. coli*: spot test	WP2(TRY-)	None	Tested at a concentration of 10% (active ingredient) in phosphate buffer saline	Reported as negative	1. Revertant count data not presented 2. Concentration of carbaryl is above solubility in water (0.01%, Carpenter et al. 1976) 3. Purity of carbaryl not given 4. Toxicity not described
Blevins et al. 1977	*Salmonella* test: plate incorporation and spot test	TA 1535 TA 1537 TA 1538 TA 98 TA 100	None	50 nmole or 11.5 ug/plate, dissolved in ethanol (approximately 95% purity)	Reported as negative	1. Revertant count data not presented 2. Brief description of protocol 3. Exact criteria for defining a negative not given 4. One concentration examined 5. No concurrent positive control data
Cook et al. 1977	*Salmonella* test: plate incorporation	TA 100	None	0.2, 2, 20, 400 ug/plate	Weakly mutagenic at 400ug/plate	1. Abstract without data 2. Data obtained by personal communication 3. Protocol was not available
DeLorenzo et al. 1978	*Salmonella* test: plate incorporation	TA 1535 TA 1537 TA 1538 TA 98 TA 100	Phenobarbital induced rats, (Sprague-Dawley) S9 liver mix	10 ug to 1500 ug/plate, dissolved in DMSO (96-99% purity)	Reported as negative with or without metabolic activation	1. Data not presented
Elespuru et al. 1974	Forward mutation to novobiocin resistance in *Haemophilus influenza*	Wild-type	None reported	0.1 mM for 1 to 9 minutes	Negative	1. Did not examine a range of concentrations 2. Purity of chemical not given 3. Exposed to chemical for a short time

(Continued on following page)

TABLE 1. (continued)

Reference	Test System	Strain	Activation System	Concentration	Result	Comment
Egert and Greim 1976	Mutation to prototrophy in E. coli: liquid suspension	K12, gal-nad-arg-NTR-	Mouse (male NMRI) liver microsomes and NADPH cofactors	Not reported	Reported as negative with or without metabolic activation	1. Insufficient information on protocol employed and revertant count data not presented 2. Purity of chemical not given 3. Chemicals tested were reported not to reduce cell survival by more than 20%
Egert and Greim 1976	Salmonella test: liquid suspension	TA 1538	Mouse (male NMRI) liver microsomes and NADPH, mice were phenobarbital induced	100 uM (solvent not reported)	Increase in mutation frequency after activation (response was weak)	1. Positive results were not confirmed by repeating experiment 2. Dose-related response not determined 3. Purity of chemical not reported
Fahrig 1974	Back mutation to prototrophy in two auxotrophic strains of Serratia marcescens, and forward mutation to galactose prototrophy in E. coli: spot test prototrophy				Reported as negative	1. Report was a review of published and unpublished data 2. Protocol and data were not given 3. Purity of carbaryl not given 4. Toxicity not described

[Continued on following page]

TABLE 1. (continued)

Reference	Test System	Strain	Activation System	Concentration	Result	Comment
Fahrig 1974	Forward mutation to streptomycin resistance in *E. coli*: liquid holding test				Reported as negative	1. Report was a review of published and unpublished data 2. Protocol and data were not given 3. Purity of carbaryl not given 4. Toxicity not described
DeGiovanni-Donnelly et al. 1968	Back mutation at indole locus of *Bacillus subtilis*	1081-	None reported	0.07% solution	Reported as negative	1. Article was brief on methodology 2. No concurrent positive controls 3. Purity of carbaryl not given 4. Concentration test of permitted 50% growth of control growth
Fiscor and Lo Piccolo 1972	Reversion to prototrophy in *E. coli*: spot test	lac-leu-cys-	None reported	Isotan insect spray with 5% carbaryl, exact concentrations used not reported	Reported as negative	1. Protocol and data not presented 2. Toxicity not described
Marshall et al. 1976	*Salmonella* test: plate incorporation	TA 1535 TA 1536 TA 1537 TA 1538	Phenobarbital induced rats (Male Sprague Dawley) S9 liver preparation	50 ug to 1000 ug/plate, dissolved in DMSO	Reported as negative with or without metabolic	1. Data presented only for 1000 ug/plate where a twofold increase above background was observed 2. Positive control data not presented 3. Purity of chemical not given

(Continued on following page)

TABLE 1. (continued)

Reference	Test System	Strain	Activation System	Concentration	Result	Comment
McCann et al. 1975	Salmonella test: plate incorporation	TA 1535 TA 1537 TA 100 TA 98	Aroclor 1254 induced rats, S9 liver mix	up to 2000 ug/plate "purest grade available"	Reported as negative with or without activation	1. Mass screening of 300 chemicals 2. Data only presented for 2000 ug/plate
McCann 1980 (personal communication)	Salmonella test plate incorporation	TA 1535	None	Analytical grade: Exper.-6/11/75 10, 100 ug/plate Experiment-9/19/73 1000, 2000, 3000 ug/plate Experiment 11/28/77- 10, 20, 50, 100, 250, 500, 1000 ug/plate	Reported as weakly positive	1. Dose-related increase
Nagy et al. 1975	Reversion to tryptophan prototrophy in E. coli: spot test	WP2 uvrA WP2	None reported	Sevin 85 exact concentration used not presented, report only indicates that either 1 to 3 mg crystal or 20-25 ul were added to each disk for the various chemicals tested	Reported as negative	1. Revertant data not presented 2. Purity of chemical not given 3. Toxicity not described

(Continued on following page)

TABLE 1. (continued)

Reference	Test System	Strain	Activation System	Concentration	Result	Comment
Rashid 1978	Salmonella test: plate incorporation	TA 1535 TA 1537 TA 1538 TA 100 TA 98	Aroclor 1254 induced rats, (male Sprague Dawley) S9 liver preparation	Analytical grade 5, 25, 25, 325, 625 ug/plate without activation 5, 10, 50, 250, 1250 ug/plate with activation	Weakly mutagenic in strain TA 1535 in the absence of metabolic activation	
Shirasu et al. 1976	Salmonella test: plate incorporation	TA 1535 TA 1536 TA 1537 TA 1538	None reported	0.02 ml of 1 mg/ml solution dissolve in DMSO	Reported as negative	1. Data not presented 2. Insufficient information on method 3. Purity of chemical not given 4. Toxicity of chemical not described 5. No concurrent positive controls
Shirasu et al. 1976	Reversion to tryptophan prototrophy in E. coli: spot test	WP2 uvrA WP2	None reported	0.02 ml of 1 mg/ml solution	Reported as negative	1. Revertant count data not presented 2. Insufficient information on method 3. Purity of chemical not given 4. Toxicity of chemical not described 5. No concurrent positive controls
Uchiyama et al. 1975	Reversion to tryptophan prototrophy in E. coli: spot test	WP2	None reported	Up to 10 mg/plate did not report exact concentrations used or solvent	Reported as negative	1. Brief description of protocol and revertant count not given 2. Purity of chemical not given 3. Toxicity not described

not been clearly established that carbaryl does not require metabolic activation to produce a mutagenic effect, the incorporation of metabolic activation by mammalian microsomal enzymes should be considered in the experimental design. As shown in Table 1, the majority of these negative tests did not use activation by an *in vitro* microsomal fraction.

Another point of concern about these negative tests is that the majority of them were spot tests (see Table 1). Relatively water insoluble chemicals are not easily detected as mutagens in a spot test assay (Ames et al. 1979). In this method, a dose of the chemical to be tested is placed on a disk of filter paper. The chemical then diffuses through the agar medium forming a concentration gradient. Because carbaryl has a low solubility in water (0.01%, Carpenter et al. 1961), it would diffuse poorly through the agar medium. An adequate concentration range may not be tested or the chemical may never leave the area of the disk, thus giving a false negative response. Also, in a spot test the sensitivity is lowered because less bacteria on the plate are exposed to the mutagenic agent at any particular dose in the resultant concentration gradient. Table 1 describes the problems with each test reported as negative. The negative results reported for the various bacterial strains do not provide substantial evidence of nonmutagenicity and do not reduce the weight of the positive results reported below.

Carbaryl mutagenicity was found only in the *Salmonella* assay using either plate incorporation testing (Rashid 1978, Cook et al. 1977, McCann 1980, personal communication) or liquid suspension testing (Egert and Greim 1976). In plate incorporation, the mutagen is mixed with the bacteria tester strain, the S9 mix, and the melted top agar and then poured on the plate. Generally, a range of concentrations are tested in a series of plates. This method allows a large population of cells to be exposed to a uniform concentration of

mutagen thus increasing the probability of detecting weak mutagenic activity. The liquid suspension method is similar to the plate incorporation assay except that the chemical to be tested is preincubated with the activation mix (S9), and the bacteria. Afterwards, the top agar is added and the mixture is poured onto plates.

In a study in which McCann et al. (1975) examined 300 chemicals, carbaryl was classified as nonmutagenic in the plate incorporation test using four Salmonella typhimurium histidine-requiring strains (Table 1). Metabolic activation did not augment the mutation frequency. A conversation with Dr. J. McCann (May 1980) revealed that subsequent more through testing of carbaryl has indicated that it appears to possess weak mutagenicity. In three independent experiments using TA 1535 (base-pair substitution sensitive), they found that carbaryl appeared as weakly mutagenic (twofold or less increase in background revertant counts) in the absence of metabolic activation. In one experiment two replicates of eight doses were examined (0 to 1000 ug/plate) and carbaryl exhibited a dose-response effect.

In general, a positive response in the Ames test is broadly defined as a reproducible, dose-related increase in the number of histidine-independent colonies (de Serres and Shelby 1979) but the precise criteria for the designation of a weak response have not been established. Presently, McCann and co-workers in collaboration with Malcolm Pike's group at the University of Southern California are developing a statistical method for analyzing dose-response data generated by chemical mutagens. When McCann and co-workers used four different models for line-fitting to determine the significance of the above dose-response data of carbaryl, they found that two of the models showed a significant ($P < 0.01$) dose-response (slope was estimated at about 3 revertant colonies/100 ug increase in dose per plate); the other two models

were rejected on the basis of a goodness-of-fit test. When this determination was carried out, excluding the two data points at 1000 ug/plate dose, on the grounds that they were artifically low due to toxicity of carbaryl at that dose, all four models were accepted by the goodness-of-fit test and a significant ($P < 0.002$) dose-response (slope was estimated to be about 7 revertant colonies per 100 ug) was shown. It should be noted that these statistical procedures are still under development, but McCann stated in a letter (1980) that any further refinements are not likely to significantly change the outcome of the basic result of carbaryl. McCann also noted that because the mutagenic response was weak, one should consider the possibility of an impurity with mutagenic activity.

The mutagenicity of carbaryl was also evaluated by Rashid (1978) employing five strains of Salmonella typhimurium (Table 1). Rashid found that carbaryl produced a weak response in only strain TA 1535 at 125 ug/plate (1.6-fold increase above the spontaneous mutation frequency) and at 625 ug/plate (1.9-fold increase above the spontaneous mutation frequency) in the absence of metabolic activation. Carbaryl in the presence of rat liver (S9) microsomal enzymes did not increase the spontaneous reversion frequency.

Cook and co-workers (1977) tested carbaryl at a concentration range of 0.2 to 400 ug/plate without metabolic activation using the base-pair substitution sensitive strain TA 100. A weak mutagenic response (twofold response over spontaneous mutation frequency) was found only at 400 ug/plate (Cook 1980, personal communication). The data could not be evaluated because the protocol was not available.

Egert and Greim (1976) investigated the mutagenic activity of carbaryl using the Salmonella typhimurium strain TA 1538 (sensitive to frameshift mutagens). These authors used a liquid suspension assay and treated bacterial

cells with 100 uM carbaryl (100% survival rate). Carbaryl was found to be slightly mutagenic without metabolic activation. The spontaneous mutation frequency of this strain was very low (< 1 revertant/10^8 cells) and the induced frequency without metabolic activation was 7 revertants/10^8 cells. After preincubation with phenobarbital-induced mouse liver microsomes and NADPH (instead of the rat S9 fraction), however, an increase in mutagenic activity (80 revertants/10^8 cells) was seen. The authors did not repeat the assay to confirm this positive response and several concentrations of carbaryl were not tested to demonstrate a dose-related effect.

In contrast, some investigators have reported negative responses with carbaryl using the Salmonella/Ames test (DeLorenzo et al. 1978, Blevins et al. 1977, Marshall et al. 1976). These negative results could be explained by solubility problems, or by cytotoxicity. Also, because the classification of a result as weakly positive or negative is somewhat arbitrary, the reported negative results may be explained by the criteria used to classify a result as negative. The reported studies of DeLorenzo et al. (1978) and Blevins et al. (1977) did not state the criteria used to define a negative result and did not provide the revertant count data to support statements of a negative response. Marshall et al. (1976) reported a twofold increase over the spontaneous rate at 1000 ug/plate as a negative result. However, revertant count data for the other concentrations tested were not provided and it was not stated if this twofold increase observed at 1000 ug/plate was reproducible.

Mammalian Cells in Culture

Ahmed and co-workers (1977a) examined the ability of 10 uM carbaryl (66% survival) to generate drug-resistant mutants in V79 Chinese hamster cells (Table 2). After carbaryl treatment and selection for mutants with 1 mM ouabain, a steroid compound which inhibits membrane Na^+/K^+ ATPase activity, these authors observed an induced mutation frequency (15.3 mutants/10^6 survivors) significantly higher than the spontaneous mutation frequency (1.8 mutants/10^6 survivors). The apparent ability of carbaryl to generate ouabain-resistant mutants would be consistent with its activity as a base-pair substitution mutagen. Although carbaryl was reported to be weakly mutagenic in this assay, several inadequacies are apparent in this report which reduce the weight of the positive result. The authors stated that the altered phenotype of five randomly picked clones was stably transmitted through many successive generations of growth in the absence of ouabain. Nevertheless, they did not present data to support this statement, i.e., the growth measurements describing the level of drug resistance acquired by these induced clones or measurements demonstrating an altered Na^+/K^+ ATPase activity. In addition, observations were not shown to be reproducible because data were derived from a single experiment. Also, the authors did not conduct experiments to show that the induced mutation frequency to ouabain resistance increased proportionally with increasing carbaryl concentrations at a fixed treatment time. Concurrent positive controls were not included in the study design and the purity of carbaryl was not described.

Drosophila

Hoque (1972) reported carbaryl to be mutagenic in Drosophila melanogaster (Table 1). The results of this study are highly questionable. Hoque exposed wild-type females to various concentrations of carbaryl (1 ppm, 5 ppm, and 10

TABLE 2. GENE MUTATION TESTS USING INSECTS AND MAMMALIAN CELLS IN CULTURE

Reference	Test System	Activation System	Concentration	Result	Comment
Ahmed et al. 1977	Forward mutation to ouabain resistance in V79 Chinese hamster cells	None	10 uM	Weakly mutagenic	1. Experiments not performed to demonstrate a dose-related effect 2. No concurrent positive controls 3. Did not demonstrate an alter Nz^{+}/K^{+} ATPase activity or growth measurement in the presence of ouabain 4. Purity of chemical not given 5. Evidence considered suggestive
Brzheskii 1972	Sex-linked recessive lethal test in Drosophila		Purity 85% 1% suspension for 24 hours	Reported as weakly mutagenic	1. Inadequate sample size 2. No concurrent positive control 3. Evidence considered suggestive
Hoque 1972	Visible mutants in in Drosophila melanogaster		1 ppm, 5 ppm, 10 ppm for 5 hours	Reported as mutagenic	1. Article brief on methodology 2. Purity of chemical not given 3. Results are considered to be invalid

ppm in the culture medium) for 5 hours. Hoque did not describe either the toxicity effects or the sterilizing effects of the three concentrations of carbaryl used in this study. In addition, inordinately high frequencies (in the F_1 generation, 69.4%; in the F_2 generation, 71.8% at 1 ppm carbaryl) of a visible variant phenotype (black eye) were observed. Such high frequencies are not characteristic of variation due to genetic alterations, and thus, in this case, phenotypic variation is most likely the result of some developmental or physiological error. In addition, Hoque described an anatomical defect (head merged with thorax) caused by carbaryl treatment, but does not state the carbaryl concentrations where this anomaly is observed or the frequency at which it occurs. Other deficiencies found in this study are: 1) insufficient sample size, 2) lack of positive control, and 3) lack of statistical analysis.

Brzheskii (1972) used the Drosophila sex-linked recessive lethal test to screen for carbaryl mutagenicity (Table 2). Drosophila melanogaster males were exposed to a 1% suspension of Sevin (85% carbaryl) in dilute sugar syrup for 24 hours. This treatment resulted in 50% survival. A small increase in the percentage of complete (heritable mutations) and partial (not all mutations transmitted to progeny) recessive lethals (0.2% $\pm$ 0.07%) compared to negative control values (0.0% experimental control, 0.05 + 0.02% in historical negative controls) was observed. If only the heritable lethals are considered, the frequency observed (0.091% $\pm$ 0.04%) is not significantly different from the spontaneous mutation values. A larger sample size would have to be examined to conclusively classify carbaryl as weakly positive or as negative.

In summary, the available evidence concerning the ability of carbaryl to cause gene mutations is considered suggestive. Additional tests are needed

which describe a dose-response curve, for example using mammalian cells *in vitro* or *Drosophila*. Furthermore, the dependence of carbaryl on metabolic activation has not been systematically explored. Thus, it is not clear whether carbaryl acts predominately as a direct mutagen or is metabolized to a genetically active chemical.

EVIDENCE CONCERNING CHROMOSOMAL EFFECTS (Studies summarized in Table 3)

Mutagenesis is not only characterized by gene mutations but also the gain, loss, or rearrangement of portions of chromosomes and the gain or loss of intact chromosomes.

Plants

Amer (1965) used root tips from *Allium cepa* (onion) to examine the ability of carbaryl to induce chromosome aberrations. He found that both formulated and "pure" (degree of purity not defined) carbaryl prepared at room temperature or at 60°C produced C-mitotic effects. C-mitosis is described as an inactivation of the mitotic spindle proceeded by a random distribution of chromosomes (Grant 1978). When onion root tips were exposed for 4 hours to 25 ppm (0.25 saturated solution) and 50 ppm carbaryl (0.5 saturated solution), multipolar anaphases, chromosome lagging, "disturbancy of metaphases" (term not clearly defined), and multinucleated cells were reported. Continuous treatment for 24 hours "nearly" arrested mitosis. This effect, however, was temporary because the root tip cells recovered after a 48-hour replacement period in water.

Amer and co-workers (1971) found similar results of carbaryl (noted as "pure" Sevin in the report, however degree of purity not defined) on mitosis of *Vicia faba* and *Gossypium barbadense* seeds. In this study, the authors

TABLE 3. CYTOGENETIC TESTS

A. Plants

Reference	Test System	Dose	Result	Comments
Amer 1965	Root tips of *Allium cepa* (onion)	85% sprayable powder (Union Carbide) 25 ppm and 50 ppm for 4 hours	C-mitotic effects; multipolar anaphases, chromosome lagging, multinucleated cells	
Amer et al. 1971	Root tips of *Vicia faba* (broad bean) and *Gossypium barbadense* (cotton)	Carbaryl described as "pure" Sevin at 25, 50, and 100 ppm for 4 hours for root treatment (seed-soak treatment was also used; 100 ppm for 6, 12, 24, and 48 hours)	Abnormal mitosis (reported as disturbancy of metaphase, chromosome lagging, tetraploid cells), frequency of induced "abnormalities" increased as treatment time or concentration was increased	1. Frequency of different "abnormalities" not reported for control cells 2. Disturbed metaphase and anaphase reported as predominant effect, this effect is defined as "chromosomes are spread irregularly over all cells"
Amer and Farah 1968	Meiotic effects studied in *Vicia faba* pollen mother cells	Two-week-old plants sprayed weekly with saturated solution of Sevin for one month, sprayed every two weeks for one month, or sprayed daily up to 8 days	Abnormal meiosis (reported as "anaphase bridges, stickiness, polyploid, lagging, fragmentation, disturbed")	1. Chromosome stickiness reported as common abnormality, fragmentation was one of the least common effects. 2. Treatment did not cause pollen sterility 3. Frequency of each abnormality not reported for control cells

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Table 3. (Continued)

A. Plants

Reference	Test System	Dose	Result	Comments
Brankovan 1972	Meiotic effects studied in corn	Seeds treated with Sevin 50 at 0.12% and 0.25% aqueous solution for 48 hours, 0.25% solution was injected into anthers of plants and treatment lasted for 6 hours	Seed treatment: dose-related increase in frequency of "chromtin" bridges (with and without fragments) Anther treatment (sporogenesis): chromosome stickiness (primary effect), C-mitotic effects	1. Russian study
Wuu and Grant 1966	Mitotic effects studied in Hordeum vulgare (barley) pollen mother cells	Sevin (purchased from Union Carbide), seed treatment at 500, 1000, 1500 ppm for 6, 12, and 24 hours	Chromosome aberrations (most common effect reported was fragments, other effects which were less frequent were chromosome lagging late telophase or interphase bridges)	1. "mutant" seedlings reported for second generation
Wuu and Grant 1967	Meiotic effects studied in Hordeum vulgare (barley) pollen mother cells	Seeds treated with Sevin 80 (purchased from Union Carbide) at 1000 ppm aqueous solution for 12 hours, plants sprayed with 500 ppm	Chromosome aberrations	1. Frequency of each aberration not reported

(Continued on following page)

Table 3. (Continued)

B. Mammalian cells in culture

Reference	Test System	Dose	Result	Comments
Ishidate and Odashima 1977	Chinese hamster fibroblast cells in vitro	(mg/ml)	Polyploid(%) 24h / 48h; Aberrant Cells(%) 24h / 48h	1. Length of treatment time not reported 2. Purity of chemical not reported 3. Toxicity of doses tested not clearly defined 4. Frequency of each particular aberration not reported 5. Data obtained by personal communication
		0.0075	0 / 2 / 2 / 1	
		0.015	0 / 1 / 12 / 24	
		0.03	0 / 1 / 29 / 35	
		control (reported in article)	0.8±1 / 0 / 1±0.9 / 1±0.6	
Kazarnovskaya and Vasilos 1977	Human embryonic fibroblasts in vitro	20, 40, and 80 ug/ml for 24 hours	Chromosomal aberrations reported	1. Russian study 2. Purity of carbaryl not reported
Vasilos et al. 1972	Human embryonic fibroblasts in vitro	20, 40, and 80 ug/ml for 6, 24, and 48 hours, technical product reported as containing 84% active ingredient	"Pathological forms of mitosis" and antimitotic effect reported	1. Russian study 2. Pathological forms of mitosis not clearly defined 3. Purity of carbaryl not reported
Shpirt 1975	Human embryonic fibroblasts in vitro	0.001, 0.01, 100, and 1000 mg/ml	Dose-dependent antimitotic effect reported	1. Russian study 2. Carbaryl purity not described 3. 1000 mg/ml resulted in 0% viability

(Continued on following page)

Table 3. (Continued)

C. Rodents

Reference	Test System	Dose	Result	Comments
Degraeve et al. 1976	Micronucleus test in mice	10^{-3} M intraperitoneally or daily for one week via intubation	Reported as negative	1. Criteria for selecting dose level not defined or toxicity of dose used not indicated 2. Purity of chemical not given
Epstein et al. 1972	Dominant lethal in mice (ICR/Ha Swiss)	Orally for 5 consecutive days at 200 mg/kg/day and at 10 mg/kg/day	Reported as negative	1. Data were not available for an evaluation
Vasilos et al. 1975a	Mitosis studied in epithelium from small intestine crypts and cornea of rats	85% commercial preparation, acute: 400 mg/kg (one half LD_{50}) 80 mg/kg, 40 mg/kg, and 20 mg/kg	Effects on mitotic spindle (mitotic arrest, C-mitosis, and chromosome fragmentation)	1. Russian study
Vasilos et al. 1975b	Mitosis studied in splenic follicles, corneal epithelium, and epithelium of glandulae intestinales of rats	85% commercial preparation, subacute: 5 and 20 mg/kg (28 administrations) orally chronic: 0.05 to 8 mg/kg/day for 6 months orally	Effects on mitotic spindle (mitotic arrest, C-mitosis, chromosome fragmentation)	1. Russian study

(Continued on following page)

Table 3. (Continued)

C. Rodents

Reference	Test System	Dose	Result	Comments
Weil et al. 1973	Dominant lethals in rats (Harlan-Wistar)	3-generation reproduction study, carbaryl was given in diet at maximum daily doses of 200 mg/kg and daily oral doses as high as 100 mg/kg	Reported as negative	1. Number of males treated not reported 2. Number of virgin females mated with each treated male not reported 3. Number of implants and fetal deaths per female of test or control groups not reported

found that the percentage of induced chromosome effects (defined as abnormal metaphases and anaphases) are dependent on the carbaryl concentration (25 ppm, 50 ppm, 100 ppm) and treatment time (4, 6, 12, 24, and 48 hours).

Amer and Farah (1968) studied the effects of carbaryl on meiosis of Vicia faba. It was found that daily spraying with 0.01% (saturated solution) of carbaryl on plants induced chromosome aberrations (chromosome stickiness, lagging, fragmentation, anaphase bridges, univalents in diakinesis, and multinucleated cells). Carbaryl treatment did not cause pollen sterility.

Similarly, Wuu and Grant (1966) induced chromosome effects in Hordeum vulgare (barley) with 500 ppm, 1000 ppm, and 1500 ppm of carbaryl. Later, Wuu and Grant (1967) found that carbaryl (80%) also affected meiosis in the mother pollen cells of barley when seeds were treated (1000 ppm for 12 hours) or when barley plants were sprayed with a aqueous solution of carbaryl (500 ppm). Meiotic effects were also reported by Brankovan (1972) in corn after treatment of the embryonic and generative phases of development with seed treatment of 0.12% and 0.25% solutions of Sevin 50 (50% carbaryl) for 48 hours and 0.25% injected into anthers for 6 hours. Tables of data were not provided in the translation of this report.

The aforementioned studies in plants indicate that carbaryl is capable of breaking chromosomes; however, it predominantly causes mitotic disturbances by interfering with the spindle mechanism in plants. This may result in chromosome segregation errors (nondisjunction).

Although the events measured in plants are similar to those in mammalian cells, their relevance to humans is sometimes questioned. It should be noted that when one examines the literature an excellent correlation is seen between the mutagenic effects demonstrated in plants and those in mammalian cells in culture (Flamm 1977).

Mammalian Cells in Culture

There is suggestive evidence that carbaryl may act as an antimitotic agent in cultured mammalian cells. The evidence comes from studies conducted in Russia. The conclusions presented in these reports are difficult to interpret because these reports only briefly describe the methodology and results, and the data expression is somewhat unconventional. Both Vasilos et al. (1972) and Shpirt (1975) found that carbaryl inhibited the mitotic activity of human embryonic fibroblasts *in vitro*. Shpirt (1975) reported that this antimitotic effect was both dependent on the concentration of carbaryl and duration of exposure. The most pronounced effects were observed at toxic levels of carbaryl. Vasilos et al. (1972) also reported an increase in "pathological forms of mitosis" of human embryonic fibroblast *in vitro* caused by 20 ug/ml, 40 ug/ml, and 80 ug/ml of carbaryl (reported as 84% active material) for 6, 24, and 48 hours, but do not clearly define these forms and only indicate that the predominant pathological form consisted of C-mitosis. Kazarnovskaya and Vasilos (1977) reported chromosome effects in human embryonic fibroblast cultures treated with 40 ug/ml and 80 ug/ml of carbaryl for 24 hours. The appearance of these aberrations was dose-dependent. Chromosome fragments were observed at 40 ug/ml, a dose which is one-half of the dose (80 ug/ml) that results in 50% growth inhibition. Chromosome and chromatid exchanges were only reported at 80 ug/ml. Ring chromosomes and inversions were not reported.

Ishidate and Odashima (1977 and personal communication 1980) studied effects of carbaryl on chromosomes of cultured Chinese hamster fibroblasts. Three different doses (0.0075, 0.015, and 0.03 mg/ml) were added to cell cultures and observations were made 24 hours and 48 hours after treatment. At the maximum effective dose, 0.03 mg/ml, several types of chromosome effects (35% aberrant cells: chromatid gaps and breaks, chromosome breaks,

translocation, ring formation, fragmentation) were observed 48 hours after treatment that were higher than in nontreated control cultures (1% aberrant cells). The report does not indicate if the high, medium, or low dose is the 50% growth inhibition dose and does not define the toxicity of the maximum effective dose. At lower doses, 0.015 mg/ml resulted in 24% aberrant cells and 0.0075 mg/ml did not appreciably affect chromosome structure (1% aberrant cells) 48 hours after treatment. The toxicity of the lower doses was not given. Although the authors stated that "gaps" were the predominant chromosomal effect, the occurrence for each particular type of aberration, or the frequency of aberrations within a cell were not given. It should be noted that gaps and breaks may be the result of cytotoxicity. A small number of cells (100) were examined in this study, thus lowering the sensitivity of the assay. The purity of carbaryl was not given in this report.

In Vivo Cytogenetic Studies

In a Russian report, Vasilos et al. (1975a) administered orally a single dose (400 mg/kg, 0.5 of LD_{50}) of commercial carbaryl (85%) to rats. The mitotic activity of cells in both the cornea and the epithelium of the small intestine were not affected at this dose. However, "pathological mitosis" (e.g., C-mitosis, fragmentation, chromosome lagging, bridges, micronuclei) were observed in the intestinal epithelium 12 to 72 hours after treatment. C-mitosis was the most pronounced effect. Significant effects were not found in the cornea cells. At lower doses, 20 mg/kg (single injection) did not affect chromosome morphology, but 40 and 80 mg/kg (single injection) caused a 1.6- and twofold increase in "pathological" forms of mitosis in intestinal epithelial cells.

Vasilos et al. (1975b) continued in vivo cytogenetic studies on rats. They found altered mitosis in intestinal epithelial cells (e.g., C-mitosis,

micronuclei, "degenerative forms," chromatid and chromosomal bridges, chromosome lagging, and fragmentation) after chronic low doses of 85% carbaryl (1 to 8 mg/kg) were given orally to rats daily for 6 months.

In contrast, Degraeve et al. (1976) did not report adverse chromosome effects in bone marrow cells or testicular cells of mice 24 hours and 48 hours after 10^{-3} M carbaryl (reported to titrate at about 90% active principle) was administered intraperitoneally or daily for one week by intubation. An increase (approximately twofold) in the incidence of diakinesis and metaphases with monovalent X and Y chromosomes in testicular cells was reported. However, the results of this study are difficult to evaluate because the report does not provide adequate information concerning the design and conduct of testing. Thus, no information was provided on the number of animals treated, and data are not presented concerning a determination of the toxicity of the test substance to mice after exposure to arrive at a maximum tolerated dose for conducting the in vivo cytogenetic assay.

Dominant Lethal Assay in Rodents

The dominant lethal assay detects chromosome damage in germ cells. Using male Swiss mice, Epstein et al. (1972) administered 1000 mg/kg and 50 mg/kg of carbaryl (reported as subtoxic) by gavage in daily portions over 5 consecutive days and reported that this dosage schedule did not produce significant early fetal deaths or preimplementation losses. Data were not presented to support this statement.

Weil and co-workers (1973) looked for dominant lethality in rats using a three generation study and found no significant lethal effects. This article is unclear on several points. The number of males treated, the number of virgin untreated females mated with each treated male, and the number of implants and fetal deaths per female of test or control groups are not given

in this report.

It should be noted that, in general, the dominant lethal assay in rodents is recognized as an insensitive test for the detection of weak mutagens because of the small number of animals used in such a study and the high background of fetal wastage observed in control animals. In addition, chromosomal effects are usually detected at higher chemical doses than are gene mutations.

In summary, the available evidence that carbaryl causes chromosome aberrations is merely suggestive. However, studies conducted in plants and studies conducted in Russian using mammalian cells *in vitro* and *in vivo* strongly suggest the ability of carbaryl to act as a spindle inhibitor. It should be noted that spindle effects were primarily reported in studies using commercial grades of carbaryl, thus the possibility of a genetically active contaminant(s) should not be ignored.

EVIDENCE INDICATIVE OF PRIMARY DNA DAMAGE
(Studies summarized in Table 4)

The reports discussed in this section describe tests which detect the ability of a chemical substance to cause primary DNA damage (for example, as manifested by DNA repair) but do not provide a measurement of mutation *per se* (Flamm 1977).

Unscheduled DNA synthesis is indicative of primary DNA damage and subsequent DNA repair. Ahmed et al. (1977b) have shown that exposure of virally transformed human cells (VA-4) in culture to carbaryl initiates unscheduled DNA synthesis at exposures as low as 1 uM as determined by photolysis of bromodeoxyuridine (BrdUrd), which is incorporated into DNA during DNA repair synthesis. These authors found that the size of the repair

TABLE 4. OTHER GENETIC EFFECTS OF CARBARYL
(Evidence indicative of primary DNA damage)

Reference	Test System	Strain	Activation System	Concentration	Result	Comment
Ahmed et al. 1977b	Unscheduled DNA synthesis as detected by autoradiography and bromodeoxyuridine phottolysis	SV-40 transform human fibroblast cells, line VA-4	Liver extracts prepared from rats/S9 mix	BrdUrd Assay- 1 to 100 uM for 24 hours autoradiography- 1 to 1000 uM for 8 hours	Positive	1. Metabolic activated did not increase genetic activity 2. Purity of chemical not given 3. Toxicity of doses used not described
Regan et al. 1976	DNA strand breaks as determined by sedimentation profiles	Human skin fibroblasts *in vitro*		100 uM for 1 hour	Negative	1. Purity of carbaryl not given 2. Toxicity of doses used not described
Siebert and Eisenbrand 1974	Gene conversion at the loci *ade-2* and *try-5* in *Saccharomyces cerevisiae*	D4		1000 ppm (4.97mM) dissolved in DMSO for 16 hours, purity 99.9%.	Negative	1. Survival at dose tested was 78%

region could be classified as UV-type repair. In addition, when the carbaryl concentration (1 uM, 10 uM, 100 uM for 24 hours) was increased, there was a concomitant increase in BrdUrd incorporation. The standard deviation or error was not given for the BrdUrd photolysis data. Ahmed and associates also used autoradiography after carbaryl treatment (1 uM, 10 uM, 100 uM, 1000 uM, for 8 hours) of human VA-4 cells as a method to detect unscheduled DNA synthesis. Positive results were obtained from this assay thus confirming the results derived from the BrdUrd photolysis assay. These authors did not present the grain numbers for all of the carbaryl concentrations examined except at 100 uM. Therefore, it is not known if a dose-related effect was found with these concentrations. Metabolic activation (liver extracts prepared from rats) of carbaryl did not enhance its ability to induce unscheduled DNA synthesis. In this study, neither the cytotoxicity of carbaryl for the concentrations employed nor the purity of carbaryl was given.

When Regan et al. (1976) treated a culture of human skin cells with 100 uM of carbaryl for 1 hour, no evidence of primary DNA damage was detected, but the cell lines used, the technique employed, and the endpoint being measured (DNA strand breaks) were not the same as that employed by Ahmed et al. (1977b). Regan determined the sedimentation profiles in alkaline sucrose gradients of cellular DNA treated with carbaryl as a method for the detection of primary DNA damage.

Another system which is indicative of primary damage to DNA includes the ability of a test agent to induce mitotic gene conversion (intragenic recombination) in yeast cells. Siebert and Eisenbrand (1974) used a diploid strain of Saccharomyces cerevisiae heteroallelic at the gene loci ade-2 and try-5 to test for the ability of carbaryl to induce mitotic gene conversion in these loci. In this organism, genetic activity or genetic damage was not

produced by a 16-hour carbaryl (1000 ppm, 99.9% purity) treatment. Yeast cells cultured in this solution of carbaryl showed only a 22% lethality. The low amount of toxicity caused by treatment with 1000 ppm of carbaryl may indicate that treatment conditions were not sufficient to induce mitotic gene conversion to a detectable level.

In summary, the results of Ahmed et al. (1977b) indicate the ability of carbaryl to interact (directly or indirectly) with DNA and the negative results reported by Regan et al. (1976) and Siebert and Eisenbrand (1974) are considered not to contradict this conclusion.

EVIDENCE CONCERNING WHETHER THE ACTIVE FORM OF CARBARYL REACHES OR AFFECTS GERMINAL TISSUE

In order for any mutagen to cause genetic alterations that may be inherited by future generations, it must reach the gonads in an active form. As articulated in the Agency's "Proposed Guidelines for Mutagenicity Risk Assessments" (Environmental Protection Agency 1980), evidence that a chemical reaches the gonads is provided by data demonstrating the alkylation of DNA or other cellular molecules, unscheduled DNA synthesis, sister chromatid exchange, or chromosome aberrations in germinal cells, and non-specific accumulation of radioactive label in the gonads following administration of the labeled chemical. When there is a lack of such data, other relevant evidence includes adverse gonadal effects following acute, subchronic, or chronic toxicity testing; and adverse reproductive effects such as decreased fertilization index, reduced sperm count, or abnormal sperm morphology.

The sperm-abnormality assay is an indicator that a chemical agent may be damaging the germ cells (Wyrobek and Bruce 1978). Wyrobek and associates (1980) analyzed semen samples from 50 carbaryl production workers and 34 new

hires who served as controls. Males exposed to carbaryl had sperm counts or sperm with fluorescent bodies (thought to be caused by meiotic nondisjunction) similar to control values. The authors also reported a higher incidence of oligospermic men (less than 20 million sperm/ml) in the exposed group (14.6%) than in the control group (5.9%). However, this difference was not statistically significant. There was a significant elevation ($P < 0.005$) of sperm abnormalities (52.0 $\pm$ 2.6%) with abnormal head morphology in currently exposed workers compared to the controls (41.9 $\pm$ 2.1). A one-tail statistical analysis ($P < 0.05$) revealed that previously exposed workers (an average of 6.3 years since last carbaryl exposure) exhibited a significant elevation of sperm abnormalities from control values (Wyrobek 1980, personal communication). Because of the sample size, however, it cannot be conclusively established if carbaryl effects are permanent. When current workers were classified as low (supervisors, foremen, maintenance personnel) and high (baggers, operators) exposure groups, both groups were shown to have significant differences in sperm abnormalities from the control group. But, there were no appreciable differences between the high and low exposure groups. In addition, there was a negative correlation between the number of years the current workers had been exposed to carbaryl and the percentage of abnormal sperm observed. Wyrobek and co-workers speculated on several mechanisms to explain this odd relationship: 1) men working longer may be exposed less because of seniority, 2) biological or pharmacological adaptation to exposure, and 3) selection for non-affected males. Although this study demonstrates a correlation between working in a carbaryl-exposed area and an alteration of human spermatozoa, it only provides suggestive evidence that these effects are due to carbaryl and/or its metabolites. It must be established that these defects of sperm morphology are not the result

of other factors, such as exposure to chemicals other than carbaryl.[1]

Whorton et al. (in press) in an earlier study, examined semen samples provided by 47 former and current workers at the same plant studied by Wyrobek et al. (1980). Similar to the findings of Wyrobek et al. (1980), the cohort with known exposure to carbaryl were found to have a higher incidence (14.9%) of low sperm counts (oligospermia) than a control group (5.5%), but this difference was not considered to be statistically significant ($P = 0.686$). Blood levels of follicle-stimulating hormone, luteinizing hormone, and testosterone were not different from control values. It should be noted that the control group was not composed of onsite controls but was composed of historical controls (workers from a composite control population with no known exposure to infertility-producing agents) collected by the Environmental Health Associates during previous chemical industry studies. These data are considered to be suggestive of a correlation between oligospermia and carbaryl exposure.

In a study performed in Russia, Krylova and Denisova (1973) examined the process of spermatogenesis in the Mongolian Tree Creeper, a small rodent. These animals inhabit an area that was sprayed with 85% Sevin wettable powder produced in America (flow rate, 2 to 2.5 kg/ha over 60 ha). One year after treatment, 9 out of 22 of the exposed animals were found to have pathological changes in the spermatozoa from the epididymis (spirally twisted tails, breaks in the neck and tail, isolated heads and tails). There was a statistically significant ($P < 0.001$) reduction in the number of spermatozoa, spermatids, and spermatocytes from the control group (tree creepers residing in an area that was not sprayed). Chemical analysis showed that 20 of the 22 exposed animals contained extremely high quantities of carbaryl in their genitalia (reported to be 0.5 to 1.5 mg/kg of body weight). The reproductive capacity

[1]See page 43 for an addendum to this study.

of the animals living in the carbaryl-exposed area appear reduced with respect to the animals that resided in an area that was not sprayed. In this article, the results were not fully interpretable because the description of the protocol was brief. Also, interpretation of results is tenuous because several factors, e.g., genetic variability of animals, duration and amount of exposure to the chemical, and the age and health of the animals, cannot be controlled in such a study.

Kitagawa et al. (1977) reported a reduction in the number of spermatogonia and spermatozoa in the testes after rats (Wister) were orally administered 3 mg of carbaryl per rat per week for one year. Only photographs of histological slides were provided in the report and not quantitative data to confirm results. Although the authors reported that the total dose used was equivalent to an acute dose representing the LD_{50}, no information concerning toxicity or the method used to determine the chronic dose level was provided. The purity of carbaryl was not reported.

Degraeve et al. (1976) reported an increase in the incidence of abnormal spermatozoa (no acrosome, abnormalities of flagellae) in the ductus deferens in carbaryl-treated male mice (10^{-3} M administered per dose daily for one week or single intraperitoneal injection). These authors, however, did not demonstrate whether exposed mice showed dose-dependent increases in the induction of abnormal sperm. A description of the toxicity of the dosage level used was not provided in the report. Carbaryl was described as commercial Sevin titrating at about 99% active principle.

Thomas et al. (1974) administered a single dose of radioactive [^{14}C] carbaryl (24 uCi/kg, 0.9 mg/kg) to Swiss-Webster albino mice. They found very small amounts of labeled carbaryl and/or its metabolites in the prostate, seminal vesicle, testes, seminal plasma, and epididymal fat. The radioactive

counts were so low in this study, it is doubtful that these counts are significantly different from background counts.

In a Russian study, Shtenberg and Rybakova (1968) found a decrease in sperm motility in albino rats at 14 and 70 mg/kg/day after 6, 9, and 12 months of carbaryl (reported as 100% active material) treatment. This effect on male fertility was dependent on the carbaryl concentration and duration of exposure. The most pronounced effect ($P < 0.001$) was observed at 70 mg/kg/day after 12 months. Significant effects ($P < 0.001$) were also observed at the lower doses after 12 months of treatment. The authors reported histological changes, e.g., oedema of interstitial tissue, reduction in the number of spermatocytes and spermatids, and destruction of germinal epithelium in rats exposed to 70 mg/kg/day for one year. Slight interstitial oedema and some depression of spermatogenesis were reported in rats exposed to 7 mg/kg/day for 1 year.

Collins et al. (1971), using a three-generation reproduction study, administered orally to female rats (Osborne-Mendel) and gerbils 10,000 ppm of carbaryl (technical grade, 99% purity). Impaired fertility ($P < 0.05$) was reported. In addition, no litters were produced from the second mating of the second generation of rats and the third generation of gerbils. It should be noted that growth depression was observed at 10,000 ppm.

In contrast, some investigators have reported no significant gonadal effects attributable to carbaryl. Dikshith et al. (1976) administered technical (99% purity) carbaryl (200 mg/kg for 3 days a week) orally to male albino rats for a period of 90 days. No histological changes were reported in the testes and epididymis. Also, these authors found no effects on the fertility of male rats. This study did not involve a quantitative assessment of the effects on sperm (e.g., sperm morphology, sperm counts). Weil et al.

(1972) observed no significant effects of carbaryl (10 mg/kg/day) on female fertility in a three-generation rat study. However, a dose of 100 mg/kg/day by intubation, which resulted in mortality, reduced fertility.

In summary, there is no direct evidence, e.g., the presence of a radioactive label of the chemical in the gonads, that carbaryl and/or its metabolite(s) reaches mammalian gonads. However, there are many reports indicating adverse gonadal and reproductive effects after carbaryl exposure. Therefore, the weight of evidence suggests that the active form of carbaryl may reach the germinal tissue of mammals.

REVIEW OF EXPERIMENTAL EVIDENCE ON THE MUTAGENICITY OF NITROSOCARBARYL

In _in vitro_ experiments, carbaryl has been shown to react with sodium nitrite under acidic conditions (pH 1) to form nitrosocarbaryl (Eisenbrand et al. 1974). Because nitrite is present in human saliva and food products, the formation of nitrosocarbaryl in stomach physiology is conceivable due to the widespread use of carbaryl. Rickard (1979) demonstrated the _in vivo_ formation of nitrosocarbaryl in the stomach of rats and guinea pigs. When guinea pigs were given either simultaneous intubation of carbaryl (1 umol) and sodium nitrite (1160 umol) or when these components were mixed with feed, approximately a 1.5% yield of nitrosocarbaryl was detected. The formation of this nitroso derivative was dependent on the amount of nitrite and the pH, and was not particularly influenced by the amount of carbaryl. Increasing the amount of carbaryl from 0.025 to 2.5 umol did not increase the yield of the nitroso-compound. In rats, where the stomach pH (3.5 to 5.5) is higher than in guinea pigs (pH 1.5), a very low yield of nitrosocarbaryl was found (0.02%) at the same concentrations of nitrite and carbaryl.

Nitrosocarbaryl has been shown to be strongly mutagenic in bacteria. Blevins and associates (1977) found that the base-pair substitution sensitive Salmonella strains TA 100 and TA 1535 were reverted by this agent without metabolic activation. The reversion frequency in TA 100 was increased by approximately 1.6 fold at 1.15 ug/plate and sixfold at 11.5 ug/plate and in TA 1535 by about threefold at 1.15 ug/plate and 76-fold at 11.5 ug/plate. Nitrosocarbaryl was not as active on the frameshift sensitive strains TA 98, TA 1537, and TA 1538. Marshall et al. (1976) found that nitrosocarbaryl increased the number of histidine-independent colonies of TA 1535 by approximately sixfold at 0.5 ug/plate and by 367-fold at 50 ug/plate without metabolic activation. These authors also found nitrosocarbaryl to be slightly active (above sixfold increase over background values) on the frameshift sensitive strains TA 1537 and TA 1538 at 50 ug/plate. Both Blevins et al. (1977) and Marshall et al. (1976) found that the mutagenic activity of nitrosocarbaryl was dose-related.

Elespuru and co-workers (1974) measured the induction to novobiocin resistance in Haemophilus influenzae. These authors found that nitrosocarbaryl was approximately an order of magnitude more potent than the well-known mutagen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). In Escherichia coli, nitrosocarbaryl was also more potent in the induction to arginine prototrophy than MNNG (Elespuru et al. 1974). Uchiyama et al. (1975) found mutagenic activity as tested by the ability to cause reversion at the tryptophan locus in Escherichia coli.

Generally, metabolic activation was not required for the mutagenic response of nitrosocarbaryl. For example, when Marshall et al. (1976) incorporated the S9 fraction in the Salmonella assay, a slight decrease in mutagenic activity was observed. Greim et al. (1977), however, found an

increase in mutagenicity after metabolic activation by mouse-liver microsomes.

Siebert and Eisenbrand (1974) reported that nitrosocarbaryl was active in causing mitotic gene conversion in Saccharomyces cerevisiae. Incubation for 2 hours on 1 ppm of nitrosocarbaryl increased the relative conversion frequency threefold for the ade-2 and fivefold for the trp-5 locus, and at 30 ppm increases were 139-fold for the ade-2 locus and 885-fold for the trp-5 locus. In this study, a dose-related effect was shown using five concentrations of nitrosocarbaryl. Regan et al. (1976) demonstrated that nitrosocarbaryl was able to induce DNA damage in cultured human cells as measured by unscheduled DNA synthesis. In addition, by using methyl labeled [^{14}C] and ring labeled [^{3}H] nitrosocarbaryl, Regan et al. (1976) found that the [^{14}C] label was associated with cellular DNA, whereas the [^{3}H] label was not. Because nitrosocarbaryl has been observed to cause reversion of base-pair substitution sensitive strains (TA 100, TA 1535), these results suggest that the nitrosocarbaryl molecule was split and the resultant methyl group alkylates DNA.

Ishidate and Odashima (1977) reported several chromosome aberrations (81% aberrant cells) in Chinese hamster cells in vitro 24 hours after exposure to nitrosocarbaryl (0.015 mg/ml). This was a significant increase compared to control values (1% aberrant cells). The toxicity of the concentration used was not given.

CONCLUSION

Although each individual study concerning the mutagenicity of carbaryl contains deficiencies, the body of evidence (i.e., data regarding both the intrinsic mutagenicity, spindle effects, and the presence of carbaryl or

metabolites in mammalian gonads) strongly suggests that carbaryl may have the potential to cause heritable genetic effects in humans. However, it should be emphasized that because carbaryl appears to act as a weak mutagen in the reported gene mutation test systems, it is likely to act as a weak mutagen in humans as well.

In the case of nitrosocarbaryl, there is evidence that it causes point mutations in bacteria and thus, it is likely to have intrinsic mutagenic activity in other organisms. If nitrosocarbaryl were to be formed or were present in humans, it might cause somatic mutations which may be involved in the etiology of cancer. Although this is an assessment of genetic risk with respect to somatic cell mutagenicity, an assessment of whether nitrosocarbaryl has the potential to cause heritable mutations in humans could not be made because there were no available data on its ability to reach or interact with mammalian germinal tissue.

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ADDENDUM

With respect to altered sperm-head morphology reported in carbaryl-exposed employees (Wyrobek et al. 1980, submitted January 6, 1981, Environ. Health Perspec.) discussed on page 32 of this document, the possibility of exposure to other chemicals was noted. Dr. H.M.D. Utidjian of Union Carbide indicated in a letter to EPA on March 21, 1981 that the workers studied by Wyrobek et al. (1980) were not occupationally exposed to other pesticides. He stated, however, that there was a potential for concomitant exposure to the reagent chemicals, alpha-naphthol and methyl isocyanate, and the solvent toluene. Exposures to methyl isocyanate were kept at very low levels (below 1 ppm). Presently, Union Carbide is collecting monitoring data in the workplace on these chemicals. With respect to the ability of these chemicals to induce sperm-head abnormalities, toluene has been reported as negative (Topham 1980) and no information was located concerning the ability of the alpha-naphthol or methyl isocyanate to alter sperm morphology.

It should be noted that the Reproductive Effects Assessment Group (REAG) made a recommendation to the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Scientific Advisory Panel on July 23, 1980, that the carbaryl-exposed workers be re-examined for altered sperm abnormalities to confirm the initial findings of Wyrobek et al. (1980) and to better define the dose-response relationship and address the question of the reversibility of the effect.

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